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1980 M N GLUKHOVA, A A TUMANOV

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A wide range of substances in environmental and other materials can be monitored through use of various microorganisms as indicators. Tests are generally based on the requirement of an organism for certain substances, sometimes due to genetic mutation, or on a sensitivity to certain substances. The microbial world is enormously diversified with regard to such properties. Advantages include the rapid multiplication of biomass seen in microbial systems and the option of using chemically defined media.		

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Considerations in the choice of organism, medium, and growth condition are discussed. Factors affecting sensitivity of the tests include inoculum size and growth phase as well as medium composition.

Quantitation is achieved through serial dilution or by measurement of growth or growth rate. Various measures of growth are available, such as turbidimetric, viable count, and determination of chemical alterations, such as enzymatic activities, respiratory processes, or pigmentation. Rapid methods of growth detection are briefly discussed.

Such methods are being adapted to problems in many fields. Substances determined by microbiological techniques include antibiotics, vitamins, amino acids, metals and trace elements, and pesticides and other toxic substances. Limits of detection are in some cases as low as 10^{-12} g/mL.

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MICROORGANISMS AS ANALYTICAL INDICATORS. EXPERIMENTAL METHODS
AND TECHNIQUES (RUSSIAN)

M. N. Glukhova and A. A. Tumanov

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An increasingly wide range of practical problems related to chemical-analytic monitoring of the environment has been solved in recent years with methods of biological indication. Different microorganisms have been successfully used as analytic indicators. They have a number of advantages over other biological objects: they are distinguished by an extremely high rate of multiplication, the biomass grows rapidly, they produce homogeneous inoculation material and are maintained relatively easily in laboratory conditions. Many representatives of the varied world of microorganisms are characterized by high sensitivity to changes in the environment and respond to the appearance of biologically active substances in the environment by the most varied response reactions which can be the basis of objective methods of analysis of the substance (Res. 1-4).

Nevertheless, biological indicators have limited applications in analytic practice, probably due to the specifically microbiological techniques of working with these objects and the features of the techniques for performing the analyses. For this reason, several questions concerning the methods of conducting an experiment based on the use of microorganisms as analytic indicators will be discussed in the present communication.

Despite the fact that the chemical composition of microbes is more or less homogeneous, their behavior toward the set of necessary nutrient substances is very different and is determined by their capacity for synthesis. This makes it possible to find selective test organisms with respect to the most varied chemical substances.

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Due to the variety of problems which can be solved by means of biological indication, the methods have recently been enriched by original procedures for bacteriological studies. We note the use of the principle of inhibition of a test microbe by antimetabolite in association with stimulation of growth by substances necessary for the normal development of the microorganisms. These compounds include amino acids, vitamins, purine and pyrimidine bases, etc., and also supplementary growth factors (Ref. 5).

Use of a highly sensitive test object is one of the fundamental events in the development of methods of biological indication of chemicals. Representatives of the most varied groups of saprophytic microorganisms are currently used as analytic indicators: gram-negative and gram-positive sporiferous and nonsporiferous bacteria, yeasts, mycelial fungi, and actinomycetes (Refs. 4, 6-8). Representatives of the genera *Bacillus*, *Micrococcus*, *Escherichia*, *Pseudomonas*, *Aspergillus*, and *Penicillium* are most frequently encountered.

Selection of the test objects is based on determination of the degree of sensitivity of microorganisms representing different systematic groups to the chemical substances investigated. An important role is played by the use of sufficiently precise methods of investigating the physiological activity of the chemical substances on one hand, and the sensitivity of the biological objects to these substances on the other hand.

The method of successive serial dilutions with growth of the microorganisms on solid nutrient media was used for performing mass

analyses in our studies (Ref. 9). The method provides reliable results and permits testing a large number of bacterial cultures in a short time. Certain criteria characterizing the sensitivity of the test organism are required for correctly evaluating the results obtained. It is usually expressed by the lowest concentration which partially or totally inhibits growth of the microbes or kills the microbes in a fixed period of time. We note that such a determination only expresses the average value of the sensitivity of the entire population of specimens of a given strain or species of microbe inoculated. Nevertheless, it is an objective criterion which permits identifying highly sensitive test organisms.

The heterogeneity of the properties of the microorganisms not only on the level of genera and species but also on the level of strains is important in selection of test objects. Interesting results are usually obtained in using auxotrophic strains, i.e., strains dependent on the presence of a certain component in the medium (Refs. 10, 11). Sporiferous microorganisms are most suitable for use as test cultures. Their use significantly simplifies and facilitates execution of biological indication analyses since an entire series of microbiological operations related to the preparation, sterilization, and distribution of nutrient media, inoculation, and growth of bacterial cultures is eliminated with ready-to-use spores. Concentrated aqueous suspensions of spores and dried spores in sealed tubes retain their viability for many months. Coccal forms of microorganisms--sarcinae and staphylococci--are also suitable test objects.

They have been successfully used in indication of antibiotics, vitamins, and amino acids and are characterized by high sensitivity to many toxic substances: organotin compounds, phosphonium salts, and other biologically active substances (Refs. 12-16).

Techniques of cultivation and preservation of the test microbes in laboratory conditions are very important for practical application of methods of biological indication. Preservation of the purity and characteristic properties of the test microorganisms is a basic problem in laboratory cultivation. The difficulty is due to the variability of microbes during cultivation on artificial nutrient media; as a result, a bacterial culture can acquire resistance to the substances studied and become unsuitable for further use as an analytic indicator. One of the necessary conditions for cultivation is maximum reduction of the frequency of inoculation. Test microbes on semi-liquid agar media can be stored in the refrigerator for several months to a year and used as inoculation material. However, storage of microorganisms in preserved form is most expedient. The methods of preservation are based on converting the cultures to an anabiotic state and storing them at low temperatures (+4 - +6°C) in dried form. Microorganisms in a state of anabiosis not only do not lose their viability, but also preserve their original properties. Lyophilized suspensions of bacterial cultures are currently widely used; they are stored for a long time in sealed tubes in a refrigerator and used directly in experiments as needed, producing a good population of microbes on multiplication (Ref. 17).

A series of factors significantly affects the results of quantitative determinations of chemical substances with microbiological methods: the composition of the nutrient medium, the age of the culture, the inoculating dose, the incubation time, and the conditions of growth (Ref. 18). One of the basic conditions for successful use of microbiological methods of analysis is the correct selection of the nutrient media for the test microorganisms; they should provide optimum conditions for the appearance of the response reaction of the microorganisms to introduction of biologically active substances in the medium.

The chemical compounds investigated can react with the components of organic media with formation of complexes whose effect is not the same as the effect of the substance investigated. Their activity with respect to the microorganisms often decreases due to formation of not very soluble compounds and precipitation of the substance into the sediments. The oligodynamic effect of heavy metal salts is significantly decreased when organic substances, especially proteins, are present in the medium. Protein media totally consist of products of organic origin: protein hydrolysates and extracts. Their composition is not only dependent on the nature of the raw material, but also on the conditions in which the substances were synthesized. It is very difficult to prevent variations in the quality of these media, since they can contain various impurities and their chemical composition is usually not completely known. In addition, media containing protein and amino acids are purified of trace element impurities

with difficulty, and for this reason, it is expedient to use liquid nutrient media with an inorganic source of nitrogen in determining them (Refs. 1, 19).

Semi-synthetic media are more constant with respect to chemical composition and are almost free of ballast substances. In most cases, they are basically prepared in the form of an acid or fermented casein hydrolysate. Due to the homogeneity of the proteins in casein and the absence of a large amount of impurities, they are preferred for use.

Use of synthetic media prepared from rigorously determined chemicals is optimum for analytic purposes. The requirements of the microbe for nitrogen, carbohydrate, and mineral nutrients, and also the supplementary growth factors and growth stimulators necessary for growth and multiplication are provided in these media by chemical compounds of known composition. These media can be liquid, semiliquid, and solid. Agar-agar, gelatin, starch, cellulose derivatives, silicate gel, and polyvinyl alcohol are used as thickeners for preparing semi-liquid and solid nutrient media (Refs. 20, 21).

Not only the chemical composition, but also physical indexes: moisture, pH, oxidation-reduction potential, etc. should be considered in selecting nutrient media. The moisture content of a solid nutrient medium and the degree of density affect the concentration of the substance tested distributed in the medium, and the pH and oxidation-reduction potential of the medium must be changed as a function of the substance investigated and the properties of the test microbe.

Use of semi-synthetic and synthetic media whose composition is rigorously known is thus most expedient for analytic purposes, but natural media prepared according to the appropriate GOSTs can also be used in some cases.

It is also necessary to consider the age of the bacterial culture used for the inoculations for obtaining constant and comparable results. Fresh 24-hour cultures are usually used for this purpose. In studying slowly growing species of microorganisms, the cultivation period is appropriately increased. This factor is less important for actinomycetes, fungi and sporiferous bacteria, since they can be used as spores.

The sensitivity of the microorganisms to different chemical substances is dependent on the inoculation dose: the sensitivity of the test microbe decreases with an increase in the inoculation dose, observed based on the decrease in the diameter of the zone of growth inhibition on nutrient agar, and vice versa, a decrease in the amount of material inoculated increases the zone, that is, the sensitivity of the microorganisms increases.

The duration of incubation and contact of microbial cultures with the substances analyzed also significantly affects the results of the studies. In some cases, the effect of the substances can be potentiated by an increase in the duration of contact of the microbial cells with the substances and the cells can be killed, while in other cases, an increase in the duration of the adaptive phase, the lag phase of growth of the microorganisms, can be observed and the microbial

cultures can develop normally and produce good growth as a result of adaptation to the unfavorable conditions. With respect to the conditions for cultivation of the test objects, they should be constant and should meet the requirements of the microbes; otherwise, inhibition or absence of microbial growth will not be an indication of the sensitivity of the microorganisms to the chemical compounds studied.

Growth reactions are most frequently used in developing microbiological methods of analysis of substances. The size of the microbial population in laboratory cultures, as well as the duration of the individual phases of the growth and development of a microorganism, are dependent on the conditions of its growth. In this respect, the number of bacterial cells, the dynamics of accumulation of biomass and the duration of the individual growth phase can quantitatively characterize the concentration of the chemical compound added to the nutrient medium (with all other conditions being equal).

The highest sensitivity of microbial cells to different environmental effects is observed in the lag phase. The more favorable the nutrient medium is for growth, the more rapidly the microorganisms begin to multiply, and the shorter the lag phase. Substitution of any component of a substrate for which there are constructive enzymes in the given species of microbe for a component requiring synthesis of adaptive enzymes causes a delay in multiplication and prolongation of the lag phase (Ref. 22). When toxic substances are added to the

nutrient medium, the duration of the lag phase can change as a function of the concentration of the substances investigated and can be used as an index of the quantitative concentration of substances.

A maximum and constant rate of cell division is a basic feature of the logarithmic phase of growth of a microbial population. The number of generations for the period of the exponential phase is an index of the growth rate of the culture and can also be used as a criterion of the quantitative concentration of chemical compounds in the medium.

Different methods of recording are used for determination of the intensity of growth of test microorganisms. They include methods of directly counting the number of microbial cells in liquid media using counting chambers, electronic counters, microscopy of stained preparations and membrane filters through which a fixed volume of the liquid tested is filtered. Growth of microbes can be determined by weighing the moist or dry biomass separated from the nutrient medium by filtration or centrifugation. The dynamics of the growth of a microbial population is also reflected by the degree of turbidity of microbial suspensions, determined by turbidimetric methods (Ref. 2). They are sufficiently precise and require a relatively short time for growth of the test microbe. However, all rules concerning sterility must be rigorously observed when they are used.

Determination of the total volume of cells by centrifugation of a microbial suspension in special tubes can be used for recording the

intensity of growth of microorganisms. The growth of bacteria is judged by the concentration of the substances contained in the microbial cells, for example, the concentration of nitrogen, as an increase in nitrogen regularly accompanies growth of bacteria (Ref. 2). It is also possible to use some physical properties of the media for this purpose: electrical conductivity, oxidation-reduction potential, viscosity, etc.

Different modifications of methods of diffusion of a substance in agar, such as the alveolar method, the disk method, and the cylinder method, are used for determining the concentration of chemicals in a solution. They are simple to perform and do not require absolute sterility in conducting the analyses (Refs. 9, 23). Diffusion methods, like turbidimetric methods, are based on the logarithmic dependence of the degree of inhibition or stimulation of growth of the test microbe on the concentration of the chemical studied. A calibration curve is constructed from the data from an analysis of standard solutions. Rapid methods based on early detection of the zones of growth inhibition of bacteria due to staining of the products of their metabolism have been developed to decrease the duration of the analysis (Refs. 9, 24).

In addition to growth reactions, various physiological-biochemical indexes of the vital activity of microorganisms can also be used for biological indication. One of the most important is enzyme activity. Three groups of enzymes are most widely used: saccharolytic, proteolytic and oxidation-reduction. Different

species of microorganisms ferment different carbohydrates, high atomic alcohols, and organic acids, with formation of such metabolites as aldehydes, acids and gaseous products. The rate of their formation and quantitative concentration in the medium can be objective criteria in developing microbiological methods of analysis of chemical compounds. For example, the rate of fermentation of carbohydrates is determined according to the amount of CO_2 formed during fermentation of the sugars in the nutrient medium by yeasts (Ref. 4). Dehydrogenase activity has been successfully used for determining toxic substances in waste waters (Ref. 25). Recording the rate of oxidation of hydrocarbons by methane-oxidizing and propane-oxidizing bacteria is used for indication of small concentrations of gaseous hydrocarbons (Ref. 4).

The intensity of respiratory processes in microorganisms is dependent on the concentration of antimicrobial substances in the medium to a significant degree. Inhibition of respiration often begins significantly earlier than the appearance of the bactericidal effect of the compounds studied, indicating the advantage of this test over reactions of inhibition of bacterial growth (Refs. 26, 27).

Microorganisms form different pigments whose synthesis is dependent on the conditions of their growth and the presence of different elements in the medium. For example, iron is necessary for synthesis of prodigiosin, and magnesium is required for synthesis of bacteriochlorophylls. A change in the color of aspergillus spores depends on the quantitative concentration of copper in the medium

(Ref. 3). In our studies, we observed acceleration of pigmentation in cultures of *Bac. mesentericus niger* and *Bac. subtilis niger* as a function of the concentration of phosphonium salts in the nutrient medium. The degree of pigmentation or rate of formation of pigments is thus an objective index of the concentration of different chemicals in the medium.

Such properties of microorganisms as luminescence, nitrogen-fixing activity, nitrification, denitrification, the intensity of different fermentations, and many other indexes can also be used for biological indication.

Microbiological methods of determining chemical substances are used in different fields of science and industry and also in a system for monitoring the natural environment which includes analysis of nature and waste waters, control of the operation of purification plants, and evaluation of the ecological systems in water and soil. For example, the high sensitivity of *Nitrosomonas* and *Nitrobacter* to pesticides permits using them as indicators of disturbances in the biological equilibrium of soil (Ref. 28). Toxic impurities in industrial waste waters are determined by recording the different indexes of the vital activity of test microorganisms (Refs. 29-31). The method of determining mercury in sea water is based on the effect of survival of bacteria of the genus *Pseudomonas* in different concentrations of salt solutions. It permits determination of mercury in amounts from $0.1 \cdot 10^{-9}$ to $0.6 \cdot 10^{-9}$ g/ml. A similar method has been developed for the analysis of mercury in food products (Ref. 32).

The quantitative concentration of trace elements in fruit juices and many other products is determined by biological indicators in the food industry (Ref. 11). Biological methods of analysis are used for determination of antibiotics in tissues and fluids (Refs. 18, 33) in medicine, and for determination of small amounts of vitamins and amino acids in biology. These methods are characterized by high sensitivity and adequate specificity. The limits of detection of antibiotics, vitamins and amino acids are 10^{-8} - 10^{-12} g/ml. The possibilities of microbiological tests are significantly increased by the capacity of microbes to produce strains with defective genes, which significantly increases their sensitivity to certain chemical compounds. Their high degree of informativeness with respect to the biological activity of various substances, relative simplicity of conducting experiments, low expenditure of labor and materials, possibility of automated analysis, determination of chemicals in small samples, etc., are some of the advantages of methods of biological indication.

In conclusion, we note that the variety of species of microorganisms, the heterogeneity of their properties, the variety of vital activities, and the methods of recording changes in these activities under the effect of chemical compounds have opened up broad possibilities and prospects for the development of the biological method as a unique direction in the analysis of chemical substances in the biosphere.

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